Journal of Medical Virology 54:271–275 (1998)

Predominance of HCV Type 2a in Saliva From Intravenous Drug Users

K.M. Roy,[1]* J. Bagg,[1] B. McCarron,[2] T. Good,[3] S. Cameron,[3] and A. Pithie[2]

[1]*University of Glasgow Dental School, Glasgow, Scotland*
[2]*Department of Infection and Tropical Medicine, Ruchill Hospital, Glasgow, Scotland*
[3]*Hepatitis Reference Laboratory, Regional Virus Laboratory, Ruchill Hospital, Glasgow, Scotland*

Paired serum and saliva samples were collected simultaneously from 50 intravenous drug users with serologically proven hepatitis C virus infection. The oral health of the volunteers was also assessed. Hepatitis C virus RNA was detected by nested PCR, employing primers from the 5′ noncoding region. Positive PCR products were sequenced using the Sequenase PCR Product Sequencing Kit (Amersham Life Sciences). HCV RNA was detected in 33 (66%) of the 50 serum samples. HCV RNA was detected in 19 (57.6%) of the corresponding 33 saliva samples. There was no correlation between oral health status or HIV seropositivity and the detection of HCV in saliva. However, subjects with HCV in their saliva were significantly more likely to complain of xerostomia ($P < 0.05$). Isolate genotypes were identified in paired serum and saliva of 15 intravenous drug users. HCV genotypes 1, 2, 3 and 6 were detected in both specimens. In seven cases, a differing HCV genotype was found in serum compared to the paired saliva specimen. The distributions of genotypes in serum and saliva were very different, with genotype 2a more common in saliva than serum ($P < 0.005$). These data suggest that in some cases the source of salivary HCV may not be serum transudation along the periodontal membrane or across damaged mucosa, and that an alternative local source, possibly the salivary glands themselves, should be considered. ***J. Med. Virol. 54:271–275, 1998.***

KEY WORDS: hepatitis C virus; genotype; serotype; oral fluid

INTRODUCTION

Hepatitis C virus (HCV) is thought to be acquired almost exclusively by percutaneous exposure. However, in as many as 40–50% of cases of HCV infection, neither a history of blood transfusion nor injecting drug use is apparent and the precise route of infection cannot be defined (Viral Hepatitis Prevention Board, 1995). This raises the possibility of less obvious modes of transmission. Early epidemiological surveys appeared to support the concept of spread of HCV by non-parenteral routes, for example sexual transmission, although studies were usually small and poorly designed, with insufficient consideration given to the exclusion of other risk factors or to the overall prevalence of HCV infection in the study population. If non-parenteral transmission occurs, the infectivity is likely to be low and large numbers of subjects would be necessary for its demonstration.

Virological studies have established the presence of HCV in body fluids other than blood, thus indicating their potential as vectors for spread of the infection [Feucht et al., 1995; Liou et al., 1992; Mariette et al., 1995; Mendel et al., 1997; Numato et al., 1993; Puchammer-Stockl et al., 1994; Young et al., 1993], yet little is known about the dynamics of such transmission. Infection by non-parenteral routes is more likely to occur if exposure is repetitive and extends over a long period of time. It is also possible that transmission occurs only at times of peak viral replication and may also be dependent on viral genotype.

The infectivity of saliva from HCV seropositive persons remains a subject of continuing interest and debate among epidemiologists, clinicians and virologists. This study aimed to further our understanding by examining the shedding of HCV in the saliva of 50 HCV seropositive intravenous drug users in relation to HIV serostatus, oral health and HCV genotype.

MATERIALS AND METHODS

Sample Population

Fifty intravenous drug users (IVDU) (17 females; 33 males, mean age 31 years, range 19–47 years) attending Ruchill Hospital, Glasgow, UK, were studied. All of the patients had been shown previously to be HCV an-

*Correspondence to: Dr. Kirsty M. Roy, Infection Research Group, Department of Oral Sciences, University of Glasgow Dental School, 378 Sauchiehall Street, GLASGOW G2 3JZ, Scotland.

Accepted 10 October 1997

tibody seropositive on the basis of screening by a second generation enzyme immunoassay (HCV EIA II System, Abbott Laboratories, Chicago) and confirmation with a recombinant immunoblot system (RIBA 2/3, Ortho Diagnostic Systems, Raritan). Twenty seven of the patients were also HIV antibody positive (9 females; 18 males, mean age 34 years, range 25–47 years).

Sample Collection and Patient Examination

Serum, whole unstimulated saliva and oral fluid (collected using the Salivette™, Sarstedt, Leicester), were collected simultaneously. All samples were aliquoted and processed within 3 hr of collection. The oral cavity was examined by torchlight illumination prior to sample collection to determine the health of the oral mucosa, the oral hygiene and the periodontal condition of the patients.

RNA Extraction and PCR

RNA was extracted from 200 μl of serum and saliva using the QIAamp blood kit (QIAGEN, Surrey, UK) according to the manufacturer's instructions, with the addition of carrier RNA (1 μg/ml) (GIBCO-BRL, Life Technologies, Paisley, Scotland) and 1 unit of RNA guard (Pharmacia, Herts, UK). Reverse transcription and PCR using primers derived from the 5′ non-coding region (NCR) was carried out as previously described [Roy et al., 1995]. Briefly, synthesis of cDNA from 10 μl RNA was carried out at 37°C for 30 min with 200 units of Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (GIBCO-BRL) in 20 μl buffer containing 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 0.1 mM (each) dNTP and 1 unit of RNA guard (Pharmacia). The RNA template was destroyed by a further incubation for 10 min at 95°C. PCR was carried out in a 100 μl mixture containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton 100, 2 units of DYNAZYME (Flowgen Instruments Ltd, Kent, UK), 0.5mM (each) outer primer, and 10μl of cDNA. Amplification occurred over 25 cycles (94°C for 36 sec, 52°C for 42 sec, and 68°C for 3 min). One microliter of the reaction mixture was amplified for a further 25 cycles with the same programme using the inner primers and the same buffer supplemented with 0.2 mM (each) dNTP. It is expected that this procedure has a detection limit of 100 genomes/ml. Amplified DNA was detected by agarose gel electrophoresis and ethidium bromide staining. In every PCR run, three negative samples were included to control for contamination of the reverse transcription, first and second round PCR reagents. HCV negative saliva spiked with HCV RNA positive serum was employed as a positive control to determine whether saliva contained any PCR inhibitors. Unfortunately no internal control, such as the β-globin gene, is available for saliva. Serum viral load was determined by the branched DNA assay version 2.0 (Chiron Corporation, Emeryville, CA, USA).

HCV Typing

HCV RNA positive samples were genotyped by direct sequencing of the PCR products using the Sequenase PCR Product Sequencing Kit (Amersham Life Sciences, Buckinghamshire, UK). DNA sequence data were compiled using PILEUP from the University of Wisconsin Genetic Computer Group programmes. Restriction Fragment Length Polymorphism (RFLP) analysis was carried out using MAPSORT from this group of programmes. Cleavage of the 5′ NCR sequence data using RsaI/HaeIII and HinfI/MvaI followed by cleavage with BstU1 or ScrF1 can distinguish genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5 and 6 [Davidson et al., 1995]. Positive serum samples were serotyped by the detection of viral type specific antibodies, using a commercially available enzyme immunoassay (HCV Serotyping 1–6 Assay; Murex, UK). This assay uses synthetic peptides representing the variable antigenic regions from the NS4 of HCV types 1–6, and is capable of identifying multiple infections.

RESULTS

HCV RNA was detected by nested PCR in the serum of 33 of the 50 IVDU (66%). The genotype distribution was as follows: 1a (n = 12); 2a (n = 4); 3a (n = 8); 6 (n = 1). Eight samples were untypeable by sequencing. HCV RNA was detected by PCR in the whole saliva and/or oral fluid from 19 of the 33 patients with HCV RNA positive serum (57.6%). The virus genotype in the salivary specimens was the same as that found in the paired serum specimen of eight IVDU: 1a (n = 4); 2a (n = 1); 3a (n = 2); 6 (n = 1). Of the remaining eleven IVDU, seven had differing genotypes in their paired serum and salivary specimens, three had an unresolved genotype in their serum and one had unresolved genotype(s) in both serum and saliva. Serotyping results were available for the 19 IVDU with HCV RNA positive paired serum and saliva specimens: 1 (n = 7); 2 (n = 1); 3 (n = 2); 1+3 (n = 1). The isolate serotype(s) of eight samples could not be resolved. Serotyping identified the same virus type as genotyping in only six patients (Table I). The presence of a mixed HCV population in those patients with differing genotypes in the paired serum and saliva specimens was not confirmed by serotyping.

Table II relates the HIV serostatus and the oral health of the 33 IVDU with HCV RNA positive serum samples to the presence of HCV RNA in their saliva specimens. The groups with and without salivary HCV were similar in relation to denture wearing, oral hygiene and the health of their oral mucosa. The presence of HCV RNA in saliva was however significantly associated with xerostomia ($P < 0.01$).

With the exception of a positive association between HIV seropositivity and oral candidiasis ($P < 0.05$), HIV co-infection did not correlate with the oral health or the presence of HCV in the saliva of the drug users. HCV RNA was present in the saliva at the same frequency

TABLE I. HCV RNA Positive Serum and Saliva Genotypes

Patient number	Serum		Saliva
	Genotype[a]	Serotype[b]	Genotype[a]
1	2a	UN[c]	2a
2	2a	UN	1a
3	3a	1	2a
4	3a	3	3a
5	1a	1	1a
6	3a	UN	2a
7	3a	1,3	3a
8	3a	2	2a
9	UN	1	2a
10	3a	UN	2a
11	1a	UN	1a
12	1a	1	2a
13	6	UN	6
14	1a	UN	1a
15	UN	1	3a
16	UN	1	UN
17	UN	UN	2a
18	3a	3	2a
19	1a	1	1a

[a]Direct sequencing of PCR product.
[b]Murex HCV Serotype 1–6 Assay™.
[c]UN = unable to type.

TABLE II. Oral Characteristics of Patients with HCV RNA Positive Serum with Respect to the Presence of HCV RNA in Their Saliva

	HCV RNA Positive saliva (n = 19)	HCV RNA Negative saliva (n = 14)
HIV positive	10	9
Negative	9	5
Oral hygiene		
Good	4	5
Moderate	6	3
Poor	9	6
Oral muscoa		
Healthy	17	12
Candidiasis	2	2
Ulceration	0	0
Lichen planus	0	0
Xerostomia		
Yes[a]	10	1
No	9	13

[a]$P < 0.01$ using Chi square.

among patients negative or positive for HIV co-infection.

DISCUSSION

Since it has been suggested that transmission of HCV may occur by non-parenteral routes, attempts have been made to identify which body fluids other than blood may be potential vehicles for transmission. This study examined the prevalence of HCV RNA in saliva and sought to determine whether this was associated with oral health, HIV serostatus or viral genotype. On the basis of earlier work [Roy, 1996], both whole saliva and oral fluid specimens were collected and analysed to ensure the greatest rate of detection of HCV in saliva. Genotyping of isolates from the 19 IVDU with both HCV RNA positive serum and saliva identified seven patients with different genotypes in their paired serum and saliva specimens. These patients may have a mixed infection, with a different predominant genotype in the two body fluids, although serotyping was unable to confirm this.

Mixed infections are likely among IVDUs, and might explain the high numbers of isolates that could not be typed. When sequencing is undertaken in a situation with a mixed infection, only the predominant sequence may prevail, or alternatively, an indeterminate sequence emerges as the mixed sequences from the different virus types appear together. A mixed infection, therefore, can only be resolved by cloning PCR products and undertaking multiple sequencing on different clones. A similar situation can occur with serotyping, where no predominant type specific antibody is discernible. Multiple exposures are inevitable within this patient group and may be responsible for the discrepancies obtained in this study when comparing the two methods of typing. Serotyping identifies viral types from both past and current infections, whereas sequencing identifies only the current predominant infecting genotype.

In Scotland, 50% of anti-HCV positive blood donors are infected with HCV type 1, most of whom carry type 1a sequences. Forty per cent are infected with type 3, all of which correspond to type 3a. Only 10% are infected with type 2, in particular with 2b, although 2a and 2c can also be found (McOmish et al., 1993; Simmonds 1994). Information to date regarding genotype distribution has been obtained purely from studies of blood donors, and the distribution may well differ within the drug-abusing community. However, the results obtained from the serum samples in the present study (by genotyping and serotyping) were broadly in agreement with this distribution, though all of the IVDU infected with type 2 carried 2a sequences. The distribution of genotypes in saliva was, however, completely different. Genotype 2, and in particular type 2a, was more common in saliva (50%) than serum (16%). Comparison of all 2a sequences obtained in this study identified point mutations. Thus, each isolate represented a distinct viral strain, ruling out the possibility of contamination with a type 2a isolate. This unexpected discrepancy between saliva and serum is difficult to explain and merits further study. Patients infected with type 2 often show a lower level of liver function abnormalities, which may indicate infection of non-hepatic areas, possibly including salivary glands [Takamatsu et al., 1992].

The presence of genotype 6 in the serum and saliva of one patient was unusual as this genotype is restricted to the Far East. An association was identified between the patient and Hong Kong, though exact details could not be obtained.

While this study indicated that HCV RNA can be detected in the saliva of at least 50% of seropositive IVDUs, the results provided some interesting data which questioned our current understanding of the source of salivary HCV RNA. There is a continual tran-

TABLE III. HCV Serum Viral Load Measured by Chiron bDNA Assay Version 2.0

Serum Viral load (RNA MEq/ml)[a]	
HCV RNA Positive saliva	HCV RNA Negative saliva
0.233	6.09
0.963	5.197
0.279	12.68
18.85	0.469
1.994	0.221
2.714	
0.308	
0.234	
0.308	
0.234	
0.353	
0.219	

T-test not significant at 95% confidence interval.
[a]HCV RNA genome equivalents $\times 10^6$ (MEq) per ml, as determined by the bDNA assay, cut-off level: 0.2 MEq/ml.

sudation of serum into the mouth through the junction of the gum margin with the tooth surface, known as the gingival crevice. The general assumption has been that HCV enters saliva by serum transudation through the gingival crevices, rather than by active replication at a local site. If this were so, we would have expected the same ratio of positive serum and saliva samples, but this was not the case. Furthermore, if serum transudate, containing virus, enters the mouth via the gingival crevices, then the presence of HCV in saliva is likely to be influenced by the number of teeth and the degree of periodontal disease. In reality, of the 19 subjects with HCV RNA positive saliva, six had less than half their adult natural teeth, of whom three had none. In these last three cases, it was clearly impossible for the virus to cross into the mouth via the gingival crevices and another source must be identified.

For transudation of HCV RNA to occur between saliva and blood, a concentration gradient must exist, with virus present in blood. Serum viral load was available for 15 patients (10 with HCV RNA positive saliva). Although the numbers are small, no significant difference was shown between the level of virus in the serum of those patients with and without detectable HCV RNA in their saliva (Table III). We have also shown previously among blood donors that the amount of virus present in blood does not correlate with its presence or absence in saliva [Roy et al., 1995].

Periodontal lesions are inflammatory, thus causing greater exudation of serum into saliva and increasing the shedding of potentially infected mononuclear cells into the salivary pool. A number of studies have confirmed that HCV replication occurs in peripheral blood mononuclear cells (PBMC) [Muller et al., 1993; Qian et al., 1992; Wang et al., 1992; Young et al., 1993]. Poor oral hygiene and periodontal disease are common among IVDUs, but in the present study showed no correlation with the presence of HCV RNA in saliva. Oral mucosal lesions are a further potential source of salivary contamination by PBMC, but most of the subjects had healthy oral mucosa with the exception of eight who showed signs of oral candidiasis. The latter, however, did not correlate with the presence of HCV RNA in saliva.

Half of the patients with HCV RNA positive saliva complained of dry mouth (xerostomia). Xerostomia was the only significant ($P < 0.01$) oral feature that differentiated patients with or without HCV in their saliva, although no particular HCV genotype was associated with dry mouth. This is an interesting finding in view of current suggestions that HCV may play a role in the aetiology of a sialadenitis similar to Sjögrens syndrome [Pirisi et al., 1994; Scott et al., 1997]. However, the result should be interpreted with some caution. Dry mouth is a common symptom and salivary secretion may be decreased by a number of factors, the most common of which is medication. All of the IVDU enrolled in the present study had been prescribed, or were abusing, at least one drug capable of reducing salivary output.

Overall, the results of this study suggest that in addition to transudation of fluid containing HCV from the general circulation into saliva, there may be other sources of HCV in saliva, possibly including active replication at the site of salivary secretion in some individuals. This would perhaps explain the lack of correlation between serum viral load and salivary excretion. Replication of HCV in salivary glands has been observed [Takamatsu et al., 1992], but further studies are needed to identify which cells harbour the virus, the viral load within saliva, and to determine whether HCV RNA positive saliva can actually transmit infection. Furthermore, the differences between the genotype of HCV present in serum and saliva highlights the problem of reliance on serum alone to identify genotypes associated with the increasingly recognised extrahepatic manifestations of HCV infection [Hadziyannis, 1997]. More work is needed to determine whether genotype specific tropism for organs and tissues other than the liver exists and to this end our group is now developing RT in situ PCR for use with salivary gland tisssue and oral mucosa.

ACKNOWLEDGMENT

This work was supported by Scottish Hospital Endowments Research Trust Grant No. 1309. K.M. Roy was funded by a Medical Research Council Studentship. Dr. D. Goldberg and Dr. E.A. Follett are thanked for their help and support in setting up the study.

REFERENCES

Chen M, Yun Z-B, Sällberg M, Schvarcz R, Begquist II , Berglund H B, Sonnerberg A (1995): Detection of hepatitis C virus RNA in the cell fraction of saliva before and after oral surgery. Journal of Medical Virology 43:223–226.

Davidson F, Simmonds P, Ferguson JC, Jarvis LM, Dow BC, Follett EAC, Seed CRG, Krusius T, Lin C, Medgysei GA, Kiyokawa H, Olim G, Duraisamy G, Cuypers T, Saeed AA, Teo D, Conradie J, Kew MC, Lin M, Nuchaprayoon C, Ndimbie OK, Yap PL (1995): Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. Journal of General Virology 76:1197–1204.

Feucht HH, Zollner B, Schroter M, Attrogge H, Leufs R (1995): Tear

fluid of hepatitis C virus carriers could be infectious. Journal of Clinical Microbiology 33:2202–2203.

Hadziyannis SJ (1997): The spectrum of extrahepatic manifestations in hepatitis C virus infection. Journal of Viral Hepatitis 4:9–28.

Liou T-C, Chang T-T, Young K-C, Lin X-Z, Lin C-Y, Wu H-L. (1992): Detection of HCV RNA in saliva, urine, seminal fluid and ascites. Journal of Medical Virology 37:197–202.

Mariette X, Loiseau P, Morinet F (1995): Hepatitis C virus in saliva. Annals of Internal Medicine 122:556.

Mendel I, Muraine M, Riachi G, El Forzli F, Bertin C, Colin R, Brasseur G, Buffet-Janvresse C. (1997): Detection and genotyping of the hepatitis C RNA in tear fluid from patients with chronic hepatitis C. Journal of Medical Virology 51:231–233.

McOmish F, Chan S-W, Dow B C, Gillon J, Frame WD, Crawford RJ, Yap P-L, Follett EAC, Simmonds P (1993): Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. Transfusion 33:7–11.

Muller H, Pfaff E, Goeser T, Kallinowski B, Solbach C, Theilmann L (1993): Peripheral blood leukocytes serve as possible extrahepatic site for hepatitis C virus replication. Journal of General Virology 74:669–679.

Numato N, Ohori H, Hayakawa Y, Saitoh Y, Tsunoda A, Kanno A (1993): Demonstration of hepatitis C virus genome in saliva and urine of patients with type C hepatitis: Usefulness of the single round polymerase chain reaction method for detection of the HCV genome. Journal of Medical Virology 41:120–128.

Pirisi M, Scott C, Fabris C, Ferracciolo G, Soardo G, Ricci P, Toniutto P, Avellini C, Vitulli D, Miotti AM, Beltrami CA, Bartoli E (1994): Mild sialadenitis: a common finding in patients with hepatitis C virus infection. Scandinavian Journal of Gastroenterology 29:940–942.

Puchammer-Stockl E, Mor W, Kundi M, Heinz F-X, Hofmann H, Kunz C (1994): Prevalence of hepatitis C virus RNA in serum and throat washings of children with chronic hepatitis. Journal of Medical Virology 43:143–147.

Qian C, Camps J, Maluenda MD, Civeira MP, Prieto J (1992): Replication of hepatitis C virus in peripheral blood mononuclear cells: effect of alpha-interferon therapy. Journal of Hepatology 16:380–383.

Roy KM, Bagg J, Bird GLA, Spence E, Follett EAC, Mills PR, Lau JYN (1995): Serological and salivary markers compared with biochemical markers for monitoring interferon treatment of hepatitis C virus infection. Journal of Medical Virology 47:429–434.

Roy KM (1996): Hepatitis C virus in saliva. PhD thesis: University of Glasgow.

Scott CA, Avellini C, Desinan L, Prisi M, Ferraccioli GF, Bardus P, Fabris C, Casatta L, Bartoli E, Beltrani CA (1997): Chronic lymphocytic sialadentitis in HCV–related chronic liver disease: comparison with Sjögren's syndrome. Histopathology 30:41–46.

Simmonds P (1994): Variability of hepatitis C virus genome. Current Studies in Hematology and Blood Transfusion 61:12–35.

Takamatsu K, Okayasu I, Koyanagi Y, Yamamoto N (1992): Hepatitis C virus propagates in salivary glands. Journal of Infectious Diseases 165:973–974.

Viral Hepatitis Prevention Board (1995): Concensus statement from consultation concerning public health priorities for the control of hepatitis C virus infection and associated disease. Viral Hepatitis 4:5–10.

Wang JT , Sheu JC, Lin JT, Wang TH, Chen DS (1992): Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. Journal of Infectious Disease 166:1167–1169.

Young K-C, Chang T-T, Liou T-C, Wu H-L (1993): Detection of hepatitis C virus RNA in peripheral blood mononuclear cells and saliva. Journal of Medical Virology 41:55–60.